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Elucidation of the Effect of Formaldehyde and Lipids on Frozen Stored Cod Collagen by FT-Raman Spectroscopy and Differential Scanning Calorimetry

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The effect of frozen storage (−10 and −30 °C), formaldehyde, and fish oil on collagen, isolated from cod muscle, was investigated. Salt- and acid-soluble collagen fractions as well as insoluble collagen indicated changes in solubility on frozen storage. Differential scanning calorimetry (DSC) showed a highly cooperative transition at 28.2 °C for isolated collagen. Changes in the thermodynamic properties of collagen were observed on frozen storage at −10 °C compared with the control at −30 °C because of changes in structure. In the presence of formaldehyde, there were no changes in the DSC collagen transition; however, in the presence of fish oil there was an increase in enthalpy and an extra peak was observed at 44.6 °C, indicating collagen−fish oil interaction. Structural changes resulted in a decrease in the solubility of collagen in salt and acid solution. FT-Raman spectra obtained for collagen at −10 °C and −30 °C confirmed the alteration of the conformation of collagen not only at −10 °C but also in the presence of formaldehyde and fish oil.

KEYWORDS: Fish fillets; cod; frozen storage; collagen extractability; amino acids; differential scanning calorimetry; FT-Raman spectroscopy

INTRODUCTION

Fish proteins are highly susceptible to biochemical changes on frozen storage leading to lower protein solubility and formation of aggregates as indicated by several investigators (*1*−*4*). These changes affect the rheological properties (*5*), which alter the texture and eating quality of fish thereby causing wastage of a scarce and rich protein source. The biochemical and rheological changes, which are pronounced at high storage temperatures and increased storage time, are reported to be due to the effects of formaldehyde, produced from the breakdown of trimethylamine oxide (TMAO) (*6*, *7*), but are more likely to be due to lipid oxidation products and large ice crystals formed in badly stored frozen fish (*8*). Thus, Badii and Howell reported that protein changes were minimized on the addition of antioxidants and cryoprotectants, even in the presence of high levels of formaldehyde (*8*).

Most studies on frozen fish have been undertaken on myofibrillar proteins, which show rapid denaturation of myosin followed by actin (*4*, *7*). However, increasing evidence suggests that connective tissue proteins such as collagen may also be denatured; this would affect muscle integrity and rheological properties of frozen lean fish fillets (*4*, *9*−*11*). Collagen is the major protein component of connective tissue which makes up about 2−5% of muscle. The amount of collagen increases toward the tail section of the fish compared with the head and can be extracted with dilute acid or neutral salt solution (*10*). The amount of collagen in fish varies with factors such as species, age, season, and nutritional status.

Collagen, a glycoprotein (MW ∼300,000 Da), has been characterized as Types 1, 11, and 111 and consists of a central triple helix with small nonhelical peptides (telopeptides) at each end (*12*). Type 1 collagen molecules are made up of three chains which may be predominantly α 1 and α 2 whereas Types 11 and 111 each contain only one chain type α 1 (11) and α 1 (111), respectively. The α chains vary depending on the tissue. Each chain forms a left-handed helix with three residues in each turn. Every third residue is glycine (30% of all amino acids), which is near the center and small enough to allow tight packing. Collagen is also unusual in that it contains high levels of imino acids, namely, proline (∼10%) and its derivative hydroxyproline (∼10%) which contribute to the helix turn (*13*). The three left-handed helices are twisted together to the right to give a coiled coil with a pitch of thirty amino acid residues at 8.5 nm. Collagen molecules are associated into fibrils, which are grouped into larger fibers that make up the extracellular matrix of the connective tissue.

Total collagen in fish is usually less than in other animals and their amino acid composition differs from that of mammalian intramuscular collagen (*14*). Collagens from cold-water fish have a smaller proportion of hydroxyproline than those of warmer-water fish and mammals, although there is more variability in different fish species compared with mammals. In addition, fish collagen has a significantly greater solubility and is less affected by the age of fish compared with animal collagen (*14*). The imino acid content also affects the denaturation temperature of molecules and the shrinkage temperature (*9*). Shrinkage in length is exhibited by collagen fibers when

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warmed at 25 °C above the body temperature, indicating breakdown or melting of the fiber structure. The denaturation temperature at which the individual molecules denature is related to the transition from coiled-coil to disordered random coils and the dissociation of the three chains. Further heating to 37 °C results in a precipitate, which is characterized by needle- or cigar-shaped "tactoids" which resemble the native fibers in appearance (*13*).

Collagen has a relatively small amount of essential amino acids and therefore has less nutritional value compared to skeletal muscle proteins (*9*, *14*). However, compared to bovine muscle collagen, fish collagen is reported to be significantly richer in some essential amino acids and poorer in hydroxyproline content (*14*). In addition, collagen contributes to the tensile strength and integrity of fish muscle, which affect the rheological properties and texture of fish products (*9*, *14*). The strength of the native fibrils is due to noncovalent H bonding and electrostatic and hydrophobic interactions as well as covalent cross-linking involving lysine and hydroxylysine (*13*). In marine fish, aggregation of muscle collagen is influenced by chilled and frozen storage (*15*, *16*). Fish collagen is less stable at higher temperatures than other species because fish proteins are adapted to lower aquatic temperatures. However, in recent years the study of fish collagen and its hydrolyzed product gelatine has gained importance as the demand for nonbovine or porcine gelatine increases because of the bovine spongiform encaphalopathy (BSE) crisis as well as for religious and social reasons.

The purpose of the present study is to characterize cod collagen by DSC, solubility changes, FT-Raman spectroscopy, and amino acid analysis during frozen storage. As the thermal activity of individual proteins varies from those present in whole flesh (*17*), connective tissue was isolated and purified to obtain pure collagen. As stated previously, changes in collagen accompany the denaturation of myofibrillar proteins in frozen storage. Therefore, the effect of factors affecting myofibrillar proteins (*8*), namely, formaldehyde, which can be produced in gadoid fish, and fish oil as well as time and temperature of frozen storage on the stability and solubility of cod collagen was investigated.

MATERIALS AND METHODS

Materials. Fresh cod fillets were purchased from J and M Sea Food, Farnham, U.K. and delivered in ice to the laboratory. Chemicals for amino acid analysis included acetonitrile HPLC grade (BDH), sodium acetate (Fisher Scientific Equipment, Loughborough, Leics, U.K.), triethylamine (TEA) phenylisothiocyanate (PITC), and amino acid standards for food analysis from Sigma-Aldrich Co. Ltd., Poole, Dorset, U.K.

Fish oil was prepared from fresh cod fillets according to Saeed and Howell (*18*). All other chemicals were Analar grade obtained from BDH/Merck Chemicals Ltd., Lutterworth, U.K. or from Sigma-Aldrich Co. Ltd., Poole, Dorset, U.K.

Collagen Preparation. Collagen was prepared and purified by the method of Montero and Mackie (*19*). Chopped fresh cod muscle (100 g) was homogenized with 400 mL NaCl solution (80 g l^{-1}) in an Omni mixer (Omni Mixer-CAMLAB), setting 8, for 1 min. The dispersion was filtered through 9-mm orifice sieve. This procedure was repeated until no muscle fibers were visible to the eye. The resultant connective tissue fibers were stirred into 500 mL 0.05 M NaCl solution containing 1 mM PMSF for 24 h at 4 °C. After centrifugation at 3000 x *g* for 15 min at 4 °C, the supernatant was discarded. NaCl solution (250 mL) was added to 25 g of the precipitate and stirred for 24 h at 4 °C followed by centrifugation at 3000 x *g* for 20 min at 4 °C. The resultant precipitate was mixed with 250 mL NaCl solution, homogenized in an Omni mixer, setting 8, for 1 min, stirred for 12 h, and finally centrifuged at 2500 x *g* for 30 min. Purified connective tissue was dried between two pieces of filter paper, divided into 1-g portions and kept at −10 °C and −30 °C for up to 40 days.

Salt and Acid-Soluble Collagen. Collagen was extracted with salt and acid solutions by the method of Borderias and Montero (*10*) to obtain soluble and insoluble fractions of collagen at intervals during frozen storage. Purified connective tissue (1 g) was homogenized with 20 mL buffer (0.03 M Tris-HCl pH 7.4) containing 0.5 M NaCl and 1 mM phenylmethylsulfonyl fluoride (PMSF) in an Omni mixer for 1 min and stirred for 24 h at 4 °C, followed by centrifugation at 7000 x *g* for 1h. This procedure was repeated twice and the supernatants added together (collagen salt-soluble fraction).

The precipitate was mixed with 0.5 M acetic acid containing 1 mM PMS, 1:20 (w/v), homogenized in an Omni mixer for 1 min, stirred for 24 h, and centrifuged at 35,000 x g for 1 h. This procedure was repeated once more and the supernatant fractions were added together (collagen acid-soluble fraction). The precipitate formed the collagen insoluble fraction. The amount of salt- and acid-soluble collagen as well as the amount of insoluble collagen were measured by the determination of hydroxyproline in each fraction.

Differential Scanning Calorimetry (DSC). Freshly prepared collagen and connective tissue from cod fillets were examined by DSC in duplicate using a Setaram DSC VII microcalorimeter (Setaram, Lyons, France). Each sample (0.80 g) and reference (water) was heated at 0.5 °C/min from 10 °C to 100 °C. Heat absorbed or released by the denatured sample results in an endothermic or exothermic peak as a function of temperature. The temperature reached when half of the protein is denatured is referred to as the transition temperature (T_m) and was measured at the tip of the peak. The total energy required to denature the protein, the enthalpy change (ΔH), was measured by integrating the area under the peak. For some samples, the experiments were repeated and the coefficient of variation of the method was less than 5%.

To study the effect of formaldehyde and fish oil on collagen, a model study was set up. Collagen from fresh cod fillets was mixed carefully with either 500 ppm formaldehyde or 500 ppm cod oil in the cold room and left overnight (18 h) at 4 °C prior to analysis by DSC. Treated collagen samples (0.80 g) were examined by DSC together with the same amount of either distilled water, distilled water + 500 ppm formaldehyde, or distilled water + 500 ppm cod oil as reference. The samples were scanned at 0.5 °C/min from 10 to 100 °C.

Amino Acid Analysis. Amino acid composition of soluble collagen fractions from fresh fish and from frozen stored fish was determined by the method of Bidlingmeyer et al. (2*0*) using the Waters Pico-Tag workstation (Water Model 712 WISP) (Waters, Watford, Herts. U.K.). Hydrolysis of soluble collagen samples (1 mg ml^{-1} protein) to yield free amino acids was undertaken by the method of Bidlingmeyer et al. (*20*, *21*). Samples were hydrolyzed with 6 N HCl (20 mL) at 110 °C for 24 h. The hydrolyzed samples and amino acid standards (20 μL) were then treated as described below. All samples were derivatized with phenylisothiocyanate (PITC) according to the Waters Pico-Tag method.

Derivatization of Amino Acids with PITC. Drying solution (20 μL), containing methanol:water:TEA (2:2:1), was added to each vacuum-dried standard and sample in tubes and vacuum-dried again. The derivatization reagent was freshly made by mixing 50 μL PITC (kept at −20 °C, under nitrogen, to prevent degradation), 350 μL methanol (HPLC grade), 50 μL TEA, and 50 μL Millie-Q water. This PITC reagent (20 μL) was added to each tube, vortex mixed, sealed, and left at room temperature for 20 min. The reagent was then removed under vacuum. The derivatized samples were vacuum-dried and dissolved in 100 μL of sample buffer (eluent A, prepared by dissolving 19.0 g of sodium acetate trihydrate in 1 L Millie-Q water, followed by the addition of 0.5 mL TEA, adjusted to pH 6.4 and filtered. To 940 mL of this solution was added 60 mL acetonitrile). Three different samples of the hydrolyzed soluble collagen fractions were analyzed, in duplicate, by reverse-phase HPLC together with amino acid standards according to the Waters Pico-Tag Amino Acid Analysis Manual, Waters Chromatography Division, 1986.

FT-Raman Spectroscopy. Fresh or thawed frozen collagen samples were examined in 7-mL glass containers (FBG-Anchor, Cricklewood, London) on a Perkin-Elmer System 2000 FT-Raman spectrophotometer with excitation from a Nd:YAG laser at 1064 nm. Frequency calibration of the instrument was performed using the sulfur line at 217 cm^{-1}.

Table 1. Amino Acid Composition of Salt- and Acid-Soluble Cod Collagen

	mean ± SD	
amino acids	salt-soluble cod collagen	acid-soluble cod collagen
asp	4.65 ± 0.2	4.95 ± 0.26
glu	5.48 ± 0.23	5.8 ± 0.35
h.p	5.16 ± 0.22	6.46 ± 0.49
ser	3.8 ± 0.17	4.92 ± 0.17
gly	28.06 ± 0.45	27.2 ± 0.87
his	0.21 ± 0.03	1.35 ± 0.16
arg	6.93 ± 0.31	9.34 ± 0.33
thr	1.42 ± 0.08	2.21 ± 0.2
ala	7.23 ± 0.37	8.6 ± 0.38
pro	7.47 ± 0.32	8.92 ± 0.27
tyr	2.51 ± 0.12	1.38 ± 0.24
val	3.86 ± 0.15	2.29 ± 0.16
met	4.22 ± 0.21	2.27 ± 0.24
cys	0.93 ± 0.03	0.3 ± 0.02
ileu	3.31 ± 0.33	2.45 ± 0.3
leu	3.1 ± 0.28	3.45 ± 0.23
phe	2.52 ± 0.16	1.47 ± 0.11
try	2.46 ± 0.22	1.77 ± 0.15
lys	6.51 ± 0.41	4.34 ± 0.24

Duplicate sets of the samples were prepared and analyzed on two different occasions using laser power of 1785 mW. The spectra were an average of 64 scans which were baseline corrected, smoothed, and normalized to the intensity of the phenylalanine band at 1004 cm^{-1} (*22*, *23*). The recorded spectra were analyzed using Grams 32 (Galactic Industries Corp., Salem, NH). Assignments of the bands in the spectra to protein vibrational modes were made on the basis of the literature (*7*, *24*, *25*).

To study the effect of formaldehyde and fish oil, collagen prepared from fresh cod fillets was mixed carefully, in the cold room, with either 500 ppm formaldehyde or 500 ppm cod oil, left overnight (18 h) at 4 °C prior to analysis by Raman spectroscopy.

Statistical Analysis. For most experiments, the mean and standard deviation were calculated and t-tests were performed to compare the different treatments. In the DSC and Raman spectroscopy, duplicate samples were used and the mean values are reported. However, for the fresh untreated collagen, four replicates were tested and the coefficient of variation was less than 2% for DSC and less than 5% for FT-Raman spectroscopy. To discriminate between treatments, the standard deviation has been assumed from the coefficient of variation of 5% for the method to calculate the upper and lower 95% confidence bands for each measurement.

RESULTS AND DISCUSSION

Amino Acids. Table 1 shows the amino acid composition of acid- and salt-soluble collagen obtained from fresh cod fillets. The amino acid profile of acid-soluble collagen was similar to that of salt-soluble collagen. The content of essential amino acids in acid-soluble collagen was similar to that reported for myocommata collagen of cod and hake (*9*). One of the important characteristics of collagen is its hydrothermal shrinking ability, which is directly related to the content of hydroxyproline (*9*, *14*). The differences in the amino acid composition, especially in the content of hydroxy amino acid, influence the structure of collagen and, interestingly, the degree of cross-linking in different types of collagen. The residues involved in helix turns are proline, hydroxyproline, and glycine. Results indicated that the imino acid (hydroxyproline and proline) content of acid-soluble protein obtained from freshly prepared cod collagen was 153.8 (residues/1000 residues) and collagen showed a denaturation temperature at 27.6 °C. Comparison of these results, with the published results (*9*) for hake, cod, and catfish acid-soluble protein, which contain 132.7, 128.3, and 118.7 (residues/1000 residues) with denaturation temperature of 19.4, 15.0, and 13.4, respectively, support the suggestion (*9*) that there is a direct positive correlation between the thermal stability of protein with their amount of imino acid content.

Table 2. Solubility of Fresh and Frozen Cod Collagen Stored at –10 and –30 °C for 40 Days

collagen	fresh	–30 °C	–10 °C
salt-soluble collagen	4.55 ± 0.23	3.45 ± 0.06	1.2 ± 0.15
acid-soluble collagen	87.63 ± 0.66	88.53 ± 0.45	76.17 ± 0.48
insoluble collagen	7.36 ± 0.22	8.0 ± 0.25	22.51 ± 0.34

Table 3. Transition Temperature °C and Enthalpy *J*/g of Fresh Cod Collagen (0 Day), Collagen Stored at –10 and –30 °C for 40 Days and Cod Connective Tissue[a]

	transition temperature °C	enthalpy *J*/g (endothermic)
fresh collagen	27.6 (±0.44)	2.75 (±0.07)
collagen at –30 °C	28.2	1.91
collagen at –10 °C	28.9	1.78
cod connective tissue	34.9	3.61

[a] Figures in parentheses refer to standard deviation based on four replicates for fresh collagen. All other figures are mean values based on duplicates.

Collagen Solubility. Solubility values for fresh and frozen collagen (**Table 2**) indicated changes in the amount of collagen soluble in neutral salt and acid solutions (pH 2.55), as a result of storage at −10 and −30 °C. At −10 °C, a significant decrease ($p < 0.05$) of 73.6% and 13.1% was observed in solubility in salt and acid solutions, respectively. In contrast, the formation of insoluble collagen increased by 205% ($p < 0.002$) compared to fresh collagen. However, insoluble collagen stored at −30 °C increased by only 8% compared to fresh collagen ($p < 0.05$). Several investigators have reported a reduction in collagen solubility because of frozen storage particularly at higher storage temperatures (*26*, *10*). Collagen insolubility may lead to changes observed in the rheological properties and toughness of frozen fish muscle (*2*, *5*, *27*, *28*).

Differential Scanning Calorimetry (DSC). Freshly prepared cod collagen (**Figure 1**) showed a transition temperature of 28.2 °C, which was 6.8 °C lower than that of connective tissue (**Table 3**). In addition, collagen enthalpy was 31% lower than that of connective tissue (ΔH value). Differences in the thermal activity of cod collagen and cod connective tissue are due to the purity of collagen, which was isolated from connective tissue (*17*). On heating collagen from 20 to 90 °C, the triple helix structure is converted to a random coil. The helix−coil transition is a highly cooperative transition, which results from the breakdown of intramolecular H bonds (*17*). The triple helix is reported to be stabilized by more than one hydrogen bond per molecule (*29*) as well as electrostatic and hydrophobic interactions.

The transition temperature (T_m) of collagen stored at −10 °C was not significantly different from that of collagen stored at −30 °C (**Table 3**). Compared with fresh collagen, the T_m increased slightly but not significantly, and the enthalpy decreased significantly ($p < 0.05$) by 35.2% and 30.5%, at −10 °C and −30 °C for 40 days, respectively (**Table 3**). Hastings (*30*) used fish connective tissue as a source of collagen and found that frozen storage at −10 °C for 2 weeks did not affect thermal stability of the sample as measured by DSC. Variation in the stability of collagen may reflect the biological variability inherent in the samples. In this context, Ledward et al. (*31*) found that tendon collagen stored at 1 °C for two weeks resulted in a large increase in the amount of collagen with a higher T_m than fresh collagen; this is in agreement with the well-established

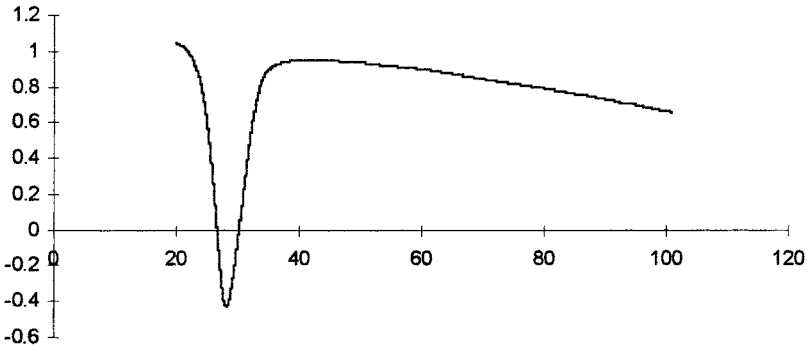

Figure 1. DSC thermogram of fresh cod collagen.

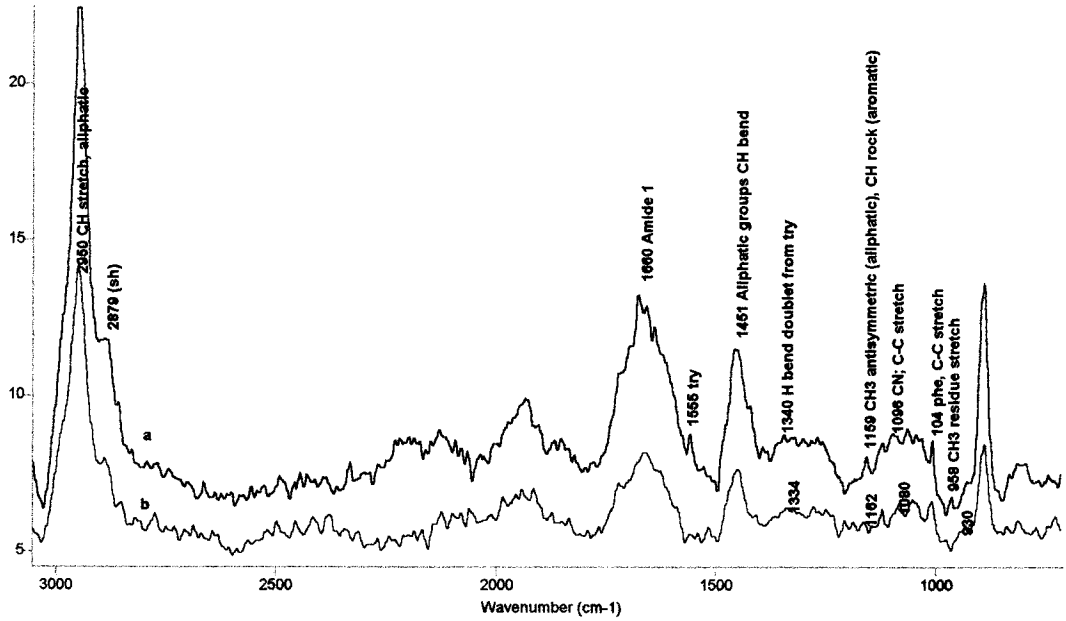

Figure 2. Raman spectra of insoluble collagen obtained from cod collagen stored at (a) –30 °C and (b) –10 °C for 40 days.

Table 4. Transition Temperature and Enthalpy *J*/g Values for Freshly Prepared Collagen Control and Fresh Collagen Stored for 18 h at 4 °C with Either 500 ppm Fish Oil or 500 ppm Formaldehyde[a]

	transition temperature °C	enthalpy *J*/g (endothermic)
fresh collagen	28.2 (±0.2)	3.46 (±0.09)
fresh collagen + 500 ppm fish oil		
peak 1	27.8	3.76
peak 2	44.6	0.04
fresh collagen + 500 ppm formaldehyde	28.1	3.42

[a] Figures in parentheses refer to standard deviation based on four replicates for fresh collagen. All other figures are mean values based on duplicates.

fact that aging collagen becomes cross-linked, and therefore increases in stability. Changes observed in the T_m and enthalpy value of frozen stored collagen at −10 and −30 °C were not only due to storage time but were also affected by freezing temperature particularly at −10 °C, which may be attributed to the cross-linking of collagen.

Surprisingly, DSC results (**Table 4**) of collagen treated with formaldehyde were very similar to the untreated control collagen sample. This is in contrast to fish protein myosin, which is very unstable and easily and irreversibly denatured with the addition of formaldehyde (*31*). Thus, formaldehyde had no significant ($p < 0.5$) effect on the T_m and enthalpy of cod collagen samples. In the presence of fish oil, the collagen transition temperature did not change significantly compared with the control ($p < 0.5$). However, the enthalpy increased by 8.7% ($p < 0.05$) and an extra peak was observed at T_m, 44.6 °C (**Table 4**), which suggests collagen−oil interaction. This finding also confirms previously published views of the authors that aggregation of fish proteins, including collagen and actomyosin, is due predominantly to the effect of lipids and lipid oxidation products in gadoid and fatty fish (*4*, *8*, *32*, *33*).

Raman Spectroscopy. As stated in the Materials and Methods section, duplicate sets of experiments were undertaken. To discriminate between treatments, the standard deviation has been assumed from the coefficient of variation of 5% (maximum for this method) and upper and lower 95% confidence bands were calculated for each measurement. Intensity values above or below these bands were considered significantly different. A shift in the wavelength ±2 is considered significantly different (*22*, *23*).

Raman spectra (3200−500 cm^{-1}) of collagen stored at −10 °C and −30 °C for 40 days indicated changes in the secondary structure of proteins (**Figure 2**, **Table 5**). Samples stored at −10 °C showed greater decrease in the intensity of the Amide I band centered at 1660 cm^{-1} compared with the control at −30 °C ($p < 0.05$) confirming changes in the protein conformation and secondary structure (*34*, *35*, *36*). However, detailed changes in the helix and coil cannot be confirmed in the present study. Increases in the peak intensity at 940, 990, and 1239 cm^{-1} were noted for samples stored at −10 compared with −30 °C ($p < 0.05$), which reflect changes in the helix, sheet, and coil,

Table 5. Relative Peak Intensity of the Raman Bands in the Region 700–3200 cm^{-1} for Cod Collagen Stored at –10 and –30 °C for 40 Days[a]

peak assignment (wavenumber ± 2 cm^{-1})	relative peak intensity	
	collagen –30 °C	collagen –10 °C
Trp (760)	0.36 (0.32, 0.40)	0.48 (766) (0.43, 0.53)
CC ring stretch and CH_2 residue rock Tyr (830, 855)	0.66 (830) (0.6, 0.72) 0.36 (855) (0.32, 0.40)	0.6 (833) (0.54, 0.66) 0.43 (859) (0.39, 0.47)
α-helix, CC stretch, CH_2 symmetric stretch (940)	0.04 (0.04, 0.04)	0.46 (0.41, 0.51)
CH_3 residue stretch (958)	0.14 (0.13, 0.15)	0.07 (930) (0.06, 0.08)
β-sheet type structure (990)	0.11 (0.10, 0.12)	0.17 (0.15, 0.19)
N; C–C stretch (1096)	1.55 (140, 170)	0.92 (0.83, 1.01)
CH_3 antisymmetric rock (aliphatic) CH rock (aromatic) (1159)	0.96 (0.87, 1.05)	0.64 (0.58, 0.70)
β-sheet type structure (1236)	0.7 (0.63, 0.77)	0.7 (0.63, 0.77)
Amide III (1245)	0.7 (0.63, 0.77)	0.4 (1248) (0.36, 0.44)
Amide III (1264)	0.8 (0.72, 0.88)	0.36 (0.32, 0.4)
Amide II (1320)	0.95 (0.86, 1.04)	0.96 (0.87, 1.05)
H bend, trp (1340)	0.95 (0.86, 1.04)	0.84 (1344) (0.76, 0.92)
aliphatic groups, CH bend (1451)	3.11 (2.81, 3.41)	2.3 (2.07, 2.53)
Amide II, COO– antisymmetric stretch (Asp), Trp (1554)	1.26 (1.14, 1.38)	0.25 (0.23, 0.27)
Amide I (1660)	4.38 (3.95, 4.81)	3.0 (2.71, 3.29)
CH stretch, aliphatic (2940)	9.8 (8.84, 10.76)	6.08 (5.48, 6.68)
shoulder (2877)	2.17 (1.96, 2.38)	1.86 (1.68, 2.04)
shoulder (2975)	3.3 (2.98, 3.62)	2.8 (2.53, 3.07)

[a] Figures in parentheses next to the assignments (or next to the intensity values if altered) refer to wavenumbers ± 2 cm^{-1}. The spectra were an average of 64 scans which were baseline corrected and normalized to the intensity of the phenylalanine band at 1004 cm^{-1}. To discriminate between treatments, the standard deviation has been assumed from the coefficient of variation of 5% for the method and upper and lower 95% confidence bands are shown for each measurement in parentheses below the intensity value.

respectively, for most proteins. The tyrosine doublet ratio (I_{850}/I_{830} cm^{-1}) increased for collagen samples stored at −10 °C (0.66) compared to −30 °C (0.5); this indicates disruption of the hydrogen bonds and exposure of residues to the outer surface of the molecule, allowing molecules to interact with water as hydrogen-bond donor or acceptor (*6*, *37*−*39*). Similar results were found for myofibrillar proteins in cod fillets stored under similar conditions (*8*).

Changes in hydrophobic groups were also noted. The Trp band at 1340 cm^{-1} shifted to 1334 cm^{-1} but there was no significant change in intensity. The Trp band at 760 cm^{-1} increased in intensity whereas the band at 1557 cm^{-1} decreased at −10 °C compared to −30 °C. Changes in the aliphatic groups were noted for samples stored at −10 °C resulting in decreased peak intensities at 1451 cm^{-1} (CH bend) and 2950 cm^{-1} (CH stretch).

Effect of Formaldehyde and Fish Oil on Collagen Structure. Raman spectra (3200−500 cm^{-1}) of collagen in the presence and absence of formaldehyde or fish oil are presented in **Figure 3**, **4**, and **Table 6**.

A decrease in the intensity of the Amide I region centered at 1660 cm^{-1} indicated an alteration in the protein secondary structure in the presence of formaldehyde or fish oil (*35*, *36*). The α-helix peak at 940 cm^{-1} did not change whereas random coil (1245 cm^{-1}) increased in the presence of formaldehyde but not significantly in the presence of cod oil. β-sheet type structures and random coil (990 and 1239 cm^{-1}) increased with both treatments ($p < 0.05$). The changes occurring during frozen storage appear to contrast those during heating of collagen in the DSC experiment in which the triple helix structure, on heating, is reported to transform to a random coil (*17*).

The tyrosine doublet ratio (I_{850}/I_{830} cm^{-1}) decreased from 1.8 for fresh collagen to 1.3 in the presence of fish oil and 1.1 in the presence of formaldehyde, indicating buriedness of residues because of interaction with fish oil or formaldehyde. A decrease in the intensity of the peak at 1340 cm^{-1} indicated exposure of buried tryptophan residues in proteins in the presence of fish oil but no significant changes were observed in the presence of formaldehyde. Further changes were observed for hydrophobic

Table 6. Relative Peak Intensity of the Raman Bands in the Region 700–3200 cm^{-1} for Insoluble Cod Collagen Obtained from Cod Collagen Stored with 500 ppm Fish Oil and 500 ppm Formaldehyde (FA) at –30 °C and –10 °C for 40 Days

peak assignment (wavenumber ± 2 cm^{-1})	relative peak intensity		
	collagen	collagen + oil	collagen + FA
Trp (760)	0.48 (0.43, 0.53)	0.4 (763) (0.36, 0.44)	0.4 (757) (0.36, 0.44)
CC ring stretch and CH_2 residue rock Tyr (830, 855)	0.25 (830) (0.23, 0.27) 0.4 (855) (0.36, 0.44)	0.51 (832) (0.46, 0.56) 0.7 (859) (0.63, 0.77)	0.9 (831) (0.81, 0.99) 1.0 (859) (0.9, 1.1)
helix C–C stretch, CH_3 symmetric stretch (937)	0.47 (0.42, 0.52)	0.92 (0.83, 1.01)	1.14 (940) (1.03, 1.25)
β-sheet type structure (990)	0.06 (0.05, 0.07)	0.2 (0.18, 0.22)	0.77 (0.69, 0.85)
Phe, ring band (1034)	0.96 (0.87, 1.05)	0.96 (0.87, 1.05)	1.96 (1037) (1.77, 2.15)
isopropyl antisymmetric stretch CN stretch (backbone) (1128)	0.47 (0.42, 0.52)	0.54 (1125) (0.49, 0.59)	1.02 (1125) (0.92, 1.12)
CH_3 antisymmetric rock (aliphatic) CH rock (aromatic) (1160)	0.38 (0.34, 0.42)	0.43 (0.39, 0.47)	0.66 (1155) (0.60, 0.72)
β-sheet type (1239)	1.52 (1.37, 1.67)	1.68 (1.52, 1.84)	2.5 (2.26, 2.75)
Amide III (random coil) (1245)	1.96 (1.77, 2.15)	1.7 (1.53, 1.87)	2.5 (2.26, 2.75)
Amide III (helix) (1264)	2.0 (1.8, 2.2)	1.8 (1259) (1.62, 1.98)	2.3 (2.07, 2.53)
Amide II (1320)	1.8 (1.62, 1.98)	1.34 (1324) (1.21, 1.47)	1.8 (1325) (1.62, 1.98)
H bend, trp (1340)	1.6 (1.44, 1.76)	1.3 (1.17, 1.43)	1.77 (1345) (1.60, 1.94)
(shoulder, residue vibration) asp, glu, lys (1425)	1.3 (1.17, 1.43)	1.6 (1.44, 1.76)	2.1 (1.89, 2.31)
aliphatic groups, CH bend (1451)	3.55 (3.2, 3.90)	3.3 (2.98, 3.62)	4.48 (4.04, 4.92)
Amide II, COO– antisymmetric stretch (Asp) Trp (1554)	0.46 (0.41, 0.51)	0.8 (1560) (0.72, 0.88)	1.37 (1560) (1.24, 1.5)
Amide I (1660)	4.29 (3.87, 4.71)	3.96 (3.57, 4.35)	5.6 (5.05, 6.15)
CH stretch, aliphatic (2940)	6.49 (5.85, 7.13)	4.7 (4.25, 5.16)	6.9 (6.22, 7.58)
shoulder (2888)	2.13 (1.92, 2.34)	1.6 (1.44, 1.76)	2.8 (2.53, 3.07)
shoulder (2976)	3.9 (3.52, 4.28)	2.76 (2.49, 3.03)	4.03 (3.64, 4.42)

[a] Figures in parentheses next to the assignments (or next to the intensity values if altered) refer to wavenumbers ± 2 cm^{-1}. The spectra were an average of 64 scans which were baseline corrected and normalized to the intensity of the phenylalanine band at 1004 cm^{-1}. To discriminate between treatments, the standard deviation has been assumed from the coefficient of variation of 5% for the method and upper and lower 95% confidence bands are shown for each measurement in parentheses below the intensity value.

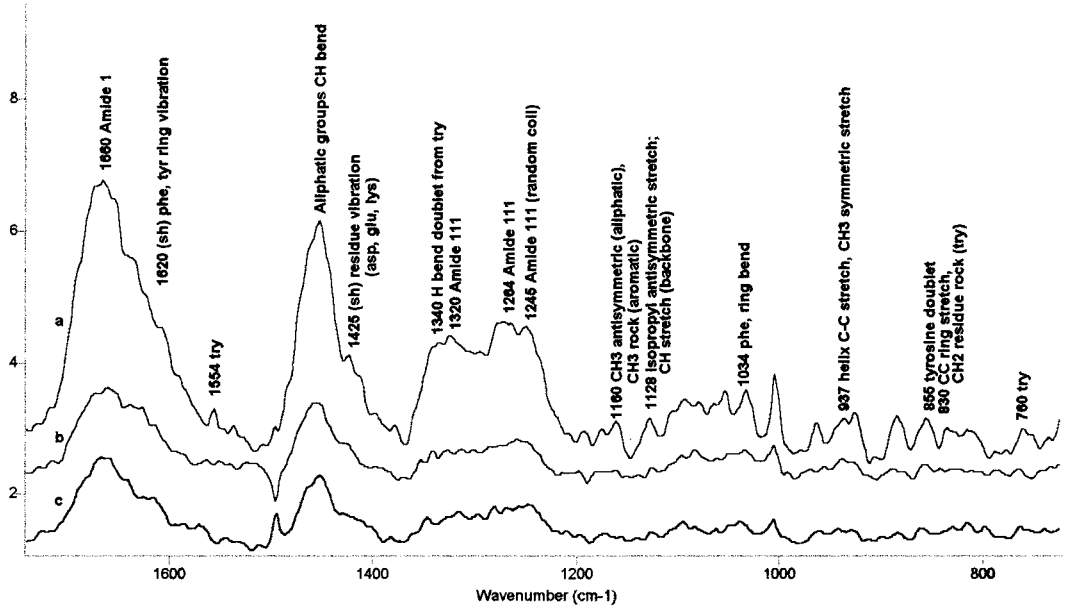

Figure 3. Raman spectra (700–1700 cm^{-1}) of (a) fresh cod collagen (b) fresh collagen treated with 500 ppm fish oil and (c) fresh collagen treated with 500 ppm formaldehyde.

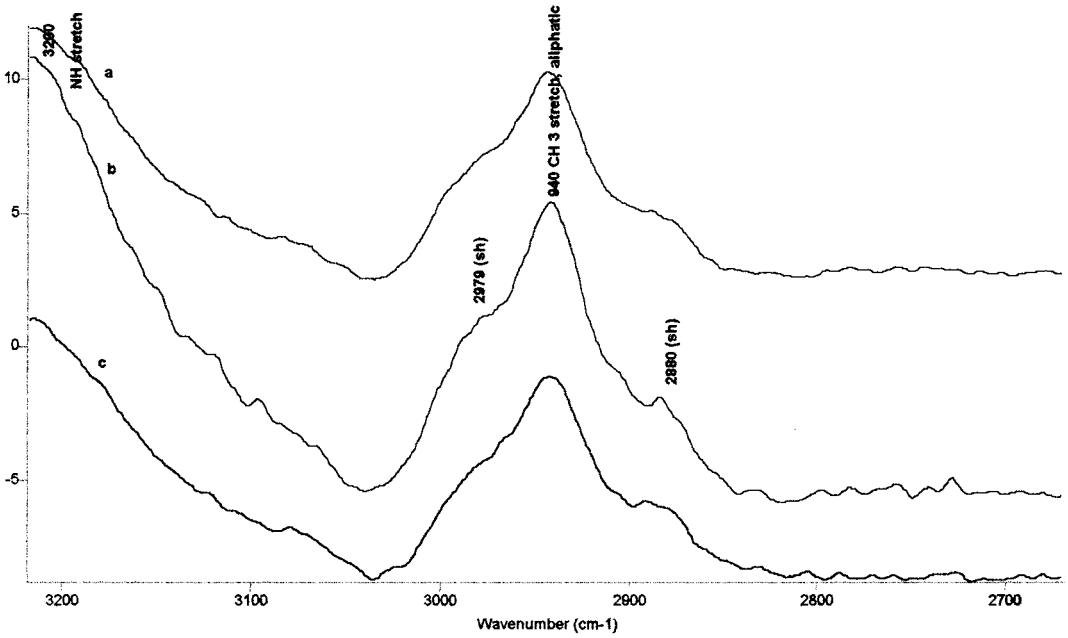

Figure 4. Raman spectra (2700–3200 cm^{-1}) of (a) fresh cod collagen (b) fresh collagen treated with 500 ppm fish oil and (c) fresh collagen treated with 500 ppm formaldehyde.

groups at CH_3 symmetric stretch, 1034 cm^{-1} Phe ring bend, isopropyl antisymmetric stretch, CN stretch (backbone) 1128 cm^{-1}, and CH_3 antisymmetric rock (aliphatic) 1160 cm^{-1} as well as Trp bands at 1554 and 1451 cm^{-1}. In the CH_3 stretch (aliphatic) region, centered at 2940 cm^{-1}, the intensity and shape of the peaks changed and a sharper peak with a sharp shoulder at 2880 cm^{-1} was observed, especially in the presence of fish oil (*40*). The results confirmed changes occurring in protein secondary structure as well as hydrophobic residues, in the presence of formaldehyde and fish oil, although the mechanism of interaction appears to be different for the two agents as confirmed by the DSC results.

CONCLUSION

Collagen significantly affects the texture and rheological properties of fish muscle; this is caused by insolubility which results from changes in the secondary structure and formation of intramolecular cross-linkages during frozen storage. Although Raman spectroscopy indicated changes in the structure of collagen in the presence of formaldehyde, changes were not observed in the thermodynamic properties as ascertained by DSC. In contrast, both the structure and thermodynamic properties of cod collagen were affected by the presence of fish oil, in a similar way to changes observed in badly stored fish at −10 °C compared to the control at −30 °C (*4*). This confirms the effect of fish oil on the structure and denaturation and cross-linking of other proteins reported by Saeed and Howell (*18*).

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